柑桔黃龍病之研究及健康管理

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摘　要

柑桔黃龍病(Huanglongbing=HLB, greening)於 1943 年由華南首次發現報告，於 1947 年在南非發生以 greening 病名發表。在台灣光復後 6 年，於 1951 年首次發現此病，經松本教授認定為新病，並命名為立枯病(Likubin)，到 1956 年此病蔓延本島各地嚴重發生，松本教授等開始其病因研究，証實為經嫁接可傳播之似病毒病害，此病於 1961 年正式在美國 Florida 召開第二屆 IOCV 國際會議由松本教授發表。病原菌以電顯切片觀察發現係韌皮部侷限，存活於篩管中不能培養之特異細菌，屬 *Candidatus* Liberibacter asiaticus，作者等以病原性及毒性之生物鑑定發現有四種系統(strain)存在於台灣，並常與萎縮病毒(CTV)及破葉病毒(CTLV)混合感染。此病造成病株葉片出現黃化斑駁(yellow mottling)病徵，樹勢衰弱終至枯萎。在分子診斷試劑開發上，經病菌基因 DNA 選殖，獲得病菌特異性 DNA 片段，而研發為一非放射性標誌之核酸探針(DNA probe)，用於點漬雜配法。之後又經定序設計引子，建立 PCR 測定法，成為快速準確檢疫技術。另再創製碘液簡易檢疫法。此系統性病害之防治一般採用綜合防治之健康管理法，包括無病柑苗之生產與種植、傳染源之撲滅及媒介昆蟲之防驅以防止病害之傳播蔓延。建立無病柑桔種苗體系為首要防治對策。頂梢微體嫁接改進法獲得之無病柑桔原種及無病原種網室之建立，可供無病芽/穗繁殖株之生產而建立其苗圃，由合格網室苗圃業者以快速繁苗法生產無病柑苗，供柑農種植。從媒介柑桔木蝨之寄主範圍中發現野生烏柑仔(*Severinia buxifolia*)為黃龍病菌之中間寄主，帶病病株成為傳染源，田間衛生以病株及中間寄主之隨時

掘除為要。流行病學研究顯示由柑桔木蝨週年密度動態、帶毒率之頻度、飛來蟲量頻度及柑株感染率與潛伏期等監測資料判定嘉義地區柳橙之感染高峰在 2 月底至 3 月間，此期為柑桔木蝨之防驅關鍵期。對罹病大柑株在發病初期(病葉比率占樹冠一半以下)以加壓注射器將 1000 ppm 四環黴素注射病株三次，具有藥療效果。按驗證法規並進行無病苗之生產與推廣為黃龍病防治之首要對策。

關鍵詞： 柑桔黃龍病、病菌系統、健康管理、無病柑桔種苗體系

緒　　言

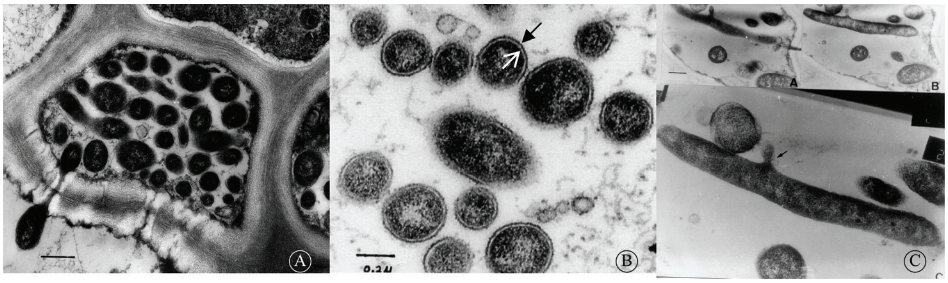
柑桔黃龍病(Huanglongbing=HLB, yellow shoot)於 1943 在中國南方已被發現[8]，而相似病害 citrus greening 於 1947 首次報告於南非[7]。在台灣於 1951 首次出現類似病害，稱為立枯病(Likubin)[18]。此病源推想應是 1945 後從華南經帶病之柑桔種苗、接穗傳入台灣。黃龍病起初認為起因於不良土壤因子例如肥份缺乏或排水不良等所引起。但經上述因素改進後，此病仍不能改善。松本等從 1956 此病流行變嚴重後開始研究此病因[18]。經嫁接傳播試驗証實此新病係病接穗傳染之似病毒性系統病害，命名為立枯病[19]。此病在 1960 年代蔓延到東南亞國家，竟在熱帶、亞熱帶柑桔產區嚴重流行為害。於 1988 在靠近台灣東北之日本沖繩 Iriomote 島也發生[20]，至 2003 續而傳播到日本鹿兒島縣之 Tokunoshima 島[11]。此病 2004 報告發生於巴西[16]，而 2005 發生於美國佛羅里達[6]。HLB 之繼續蔓延對現今之世界柑桔產業構成嚴重為害之威脅。屬熱敏感型之南非 HLB 菌在涼溫(22-24℃)發病。屬耐熱型之亞洲型 HLB 菌在高溫(27-32℃)及涼溫均能發病為害，而比南非型發病嚴重，縮短樹齡短於 10 年，嚴重減低產量及果實品質，近幾十年在熱帶、亞熱帶之亞太地區流行發生。為發展防治技術近數十年來在台灣推展 HLB 之病因、偵測、流行病學及病害管理等研究[1, 2, 3, 4, 19, 27]。黃龍病菌往往與萎縮病毒(CTV)及破葉病毒(CTLV)混合感染引起嚴重黃化斑駁而衰萎病徵，終至植株枯萎。此系統性病害經無性繁殖苗傳播以及媒介木蝨昆蟲於田間傳播，通常以綜合防治法為防治策略。無病種苗之建立係首要防治對策。

病因研究

立枯病(LCB)病株呈全株黃萎病徵與生理病相似，早期認為由不良土壤因素所引起，但經土壤與施肥、微量元素施用與排水改良等均未能防治改善此病。吾等從1956年起進行病因研究[18]。經病接穗/芽之嫁接接種試驗証實此病為似病毒病害，且發現病株經常檢測到萎縮病毒(CTV)[19]。後來發現健康柑株也往往有CTV感染，但CTV單獨感染不會引起HLB病徵。而接種HLB病穗/芽之枳殼株(CTV免疫品種)，再將其病穗/芽接種到健康柑苗亦引起HLB典型病徵，証實病株為混合感染，病原不單是CTV。著者(1968)發現本病引起典型病徵與中國黃龍病之病徵極為相似[8]，因而推定立枯病(LCB)之病原與黃龍病(HLB)之病原相同[18, 27]。Calavan(1968)亦曾推想台灣之立枯病與中國大陸之黃龍病(yellow shoot)、南非之greening及美國之stubborn有很多相似特徵。

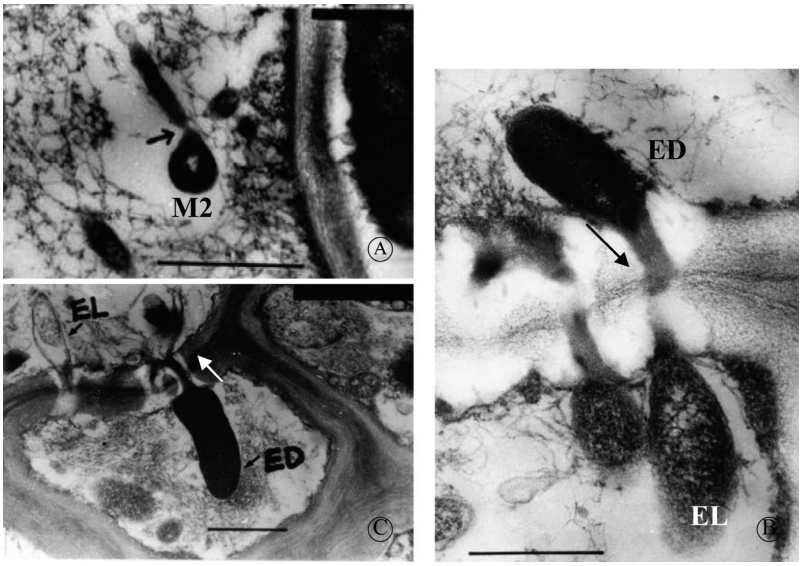
1. 病原菌之電子顯微鏡觀察

病原菌之電顯觀察研究由研究生黃安利於1983-1987間完成[3]，經病葉脈超薄切片之電顯觀察發現黃龍病菌生存於篩管中之原核菌(圖一, A)，經系列切片之立體構造觀察，此菌多形態性，菌體橫切面呈圓形而胞膜由外側細胞壁(7.5 nm)及內側原形質膜(7.5 nm)兩層所構成，20-25 nm厚，因此屬細菌類(圖一, B)，其成熟體呈桿狀，大小350-500×660-1500 nm(圖一, C)。對此菌曾進行培養試驗，但不能成功，發現此菌屬難能培養之營養苛求細菌，似絕對寄生菌。其菌體通常以出芽方式(budding)繁殖(圖二, A)，由成熟菌體母細胞芽生幼生長體，可彎曲成細長桿狀(100-250×500-2500 nm)，含濃細胞質，脫離母細胞成熟成桿狀。菌體在篩管中經篩孔移動，擠壓變形穿通篩孔，以細胞質稀薄一頭為前端而胞質高密度尾部為後端(圖二, B&C)。屬亞洲型之HLB病菌命名為*Candidatus* Liberibacter asiaticus [6, 28]。



圖一、柑桔黃龍病(HLB)特殊難養細菌(HLB-FB)之電子顯微鏡觀察。

Fig. 1. (A) Electron micrograph of Citrus Huanglongbing (HLB) fastidious bacteria (HLB-FB) packed in sieve tube of Ponkan mandarin. Large ones are mature bodies, and smaller ones are young bodies. Bar = 500 nm. (B) Electron micrograph showing cross-section of FB bodies surrounded by two-layered envelope (←), 20-25 nm thick consisting of a cell wall and inner cytoplasmic membrane (7.5 nm thick / each). (C) Serial cross sections of lateral vein from diseased Ponkan leaf (A&B), and C) a stereomicrograph of piling up with A and B sections, showing a rigid rot of nature FB body with budding (←). Bar = 500 nm.



圖二、(A) 柑桔黃龍病菌之出芽方式繁殖及細菌體貫通篩孔之情形。菌體在桶柑葉(B)及椪柑葉中(C)。

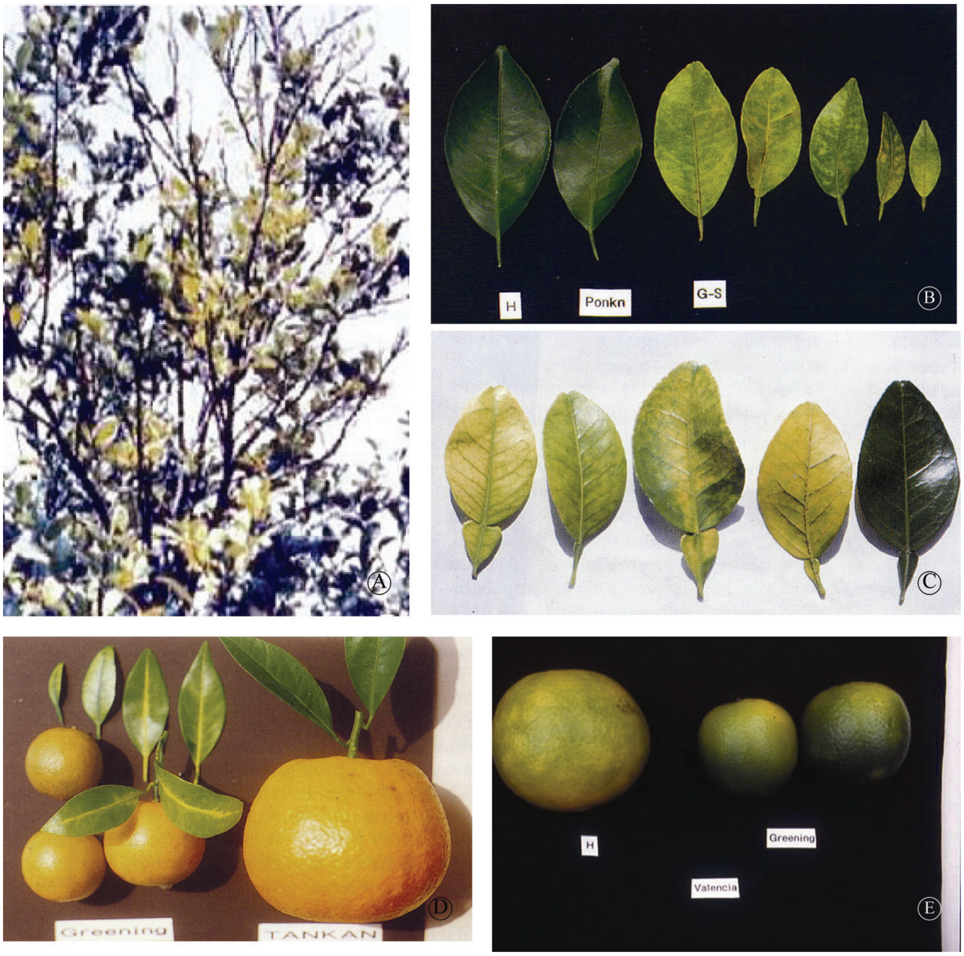
Fig. 2. (A) Side-budding (↓) (M2) of two mature bodies by producing slender rods of young daughter cell. (B) Tankan leaf, showing electron-dense posterior anterior at ention side and electron-lucent (EL) at the exit side of sieve pore. (C) Sie-pore passage (→) of FA bodies in sieve tube of disease Tankan leaf showing electron-dense posterior (ED) with dense cytoplasm at the entrance side and electron-lucent anterior.

2. 病徵

此病病徵因柑桔品種、病菌系統及環境因子等不同而略顯差異。一般病徵呈現葉脈與邊緣組織之黃化而後進展全葉變為帶淡綠斑駁之黃萎[18] (圖三, B, C)。隨老熟病葉硬化、向外捲曲而往往葉脈隆起呈木栓化，病葉提早脫落，而後新生葉變細小帶有缺鋅狀及黃萎等病徵，最終引起枝梢枯萎(圖三, A)。病株緩慢生長變矮化、異常開花包括提早開花隨後落花與落果，殘留少數果實萎縮、果皮轉黃熟不良而維持部份綠色，即所謂greening病名之由來。經病葉切片之顯微鏡觀察到韌皮部細胞之增生及其中澱粉粒堆積等內部病徵。

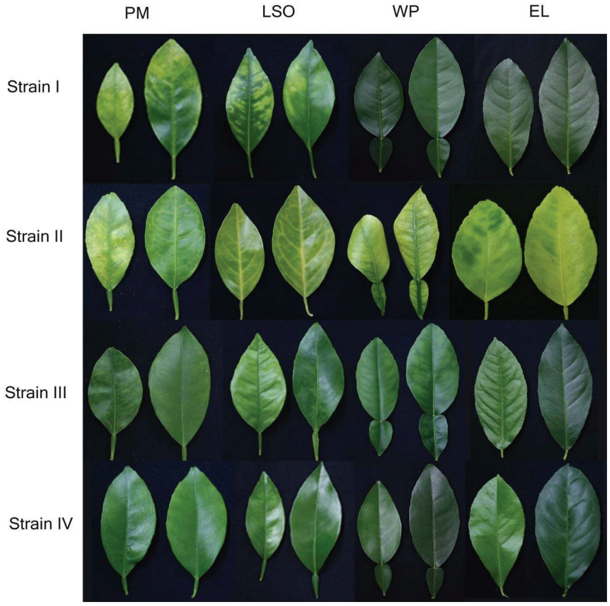
3. 黃龍病病原細菌之演化系統

柑橘黃龍病自1951年在台灣北部發生後，初期只危害國內主要柑橘品種如椪柑、桶柑、及柳橙等，1971 後開始發現感染柚類，並逐步擴及所有柑橘品種，顯示此病菌系統近幾十年來發生演化。於病原細菌的系統鑑定，以無病毒複合感染之黃龍病菌分離株，對四種鑑別柑橘品種(包括椪柑、柳橙、文旦與檸檬)進行生物檢定，根據其病原性與毒性鑑別出目前台灣田間之黃龍病菌系統至少有四種：系統 I 僅對椪柑與柳橙具高病原性並產生典型黃龍病病徵，為原始系統；系統 II 對四種鑑別品種皆具高毒性，產生典型的黃化病徵，並可快速增殖其內；系統Ⅲ 僅在椪柑與柳橙引起中度病徵，在文旦出現輕度病徵；系統 IV 為弱毒系統，僅感染椪柑及柳橙，幾無病徵表現，於PCR測定值亦輕微[4, 29] (表一, 圖四)。Tomimura, K等人(2008)將沖繩、台灣及東南亞國家的黃龍病菌分離株做分子上的親緣關係比較。分析結果發現bacteriophage-type DNA polymerase (DNA pol)的區域序列在所有的分析分離株有明顯的差異，而可區分A, B及C三群。台灣的第I型病菌系統屬於A群，第II型系統屬於B群，而沖繩的菌體分離株卻沒有DNA pol區域[28]。因此未來可參考DNA pol區域的序列設計可區分黃龍病菌系統的鑑別性引子對。



圖三、黃龍病病株之病徵(A)黃化病枝之梢枯,(B)椪柑之病葉呈現萎黃帶綠斑駁病徵,(C)柚葉之萎黃病葉呈現脈葉木栓化, (D)桶柑病果之縮果病徵, (E)柳橙之病果呈現黃熟轉色不均維持果皮綠色。

Fig. 3. (A) Severely HLB-affected trees, showing severe yellowing symptom with dieback and ultimately death of entire plants. (B) Leaf symptoms of HLB-affected Ponkan mandarin, showing bright yellowing with pale green mottling of mature leaves (center two), and newly grown small and slender leaves with interveinous chlorosis resembling zinc-deficiency symptoms (right 3). (C) Leaf symptom of diseased Wentan pummelo, showing bright-yellow mottling and vein corking on mature leaves compared with healthy leaf (right). (D) Leaf and fruit symptoms of diseased Tankan tangor, showing yellowing of veins and adjacent tissue, and smaller fruits with atrophy and pate green, compared to a healthy fruit with bright orange color (right). (E) Fruit symptoms of diseased Valencia orange, showing atrophy and poor color turning of mature fruits remaining green (right 2), compared with a healthy fruit (left).



圖四、柑桔黃龍病菌系統在四種不同柑桔上造成不同病徵及罹病程度。

Fig. 4. Different type and severities of the symptoms induced by the 4 HLBB strains (I~IV) in four differential citrus cultivars of Ponkan mandarin (PM), Liucheng sweet orange (LSO), Wentan pummelo (WP) and Eureka lemon (EL) under greenhouse conditions during 2005~2006.

表一、黃龍病系統及病原性特徵以病徵級數與 PCR 測定值表示

Table 1. The pathogenicity characterization of HLB-FB isolates according to disease index and PCR detection of HLB-FB infection

Strain	Differential cultivars of citrus[a]			
	PM	LSO	WP	EL
I	3 /+++[a]	3 / +++	0 / -	0 / -
II	3 / +++	3 / +++	3 / +++	3 / +++
III	2 / ++	2 / ++	1 / +	0 / -
IV	0 / +	0 / +	0 / -	0 / -

[a] Disease index / PCR index. Disease index was graded 12 months after inoculation: 0, healthy looking without symptoms; 1, mild chlorotic symptoms; 2, intermediate symptoms including chlorosis, mottling with intermediate dwarfing; 3, typical greening symptoms including leaf yellow mottle and curling, vein-yellowing with distinct dwarfing. PCR index, pixel value (density count) of the HLB-FB-specific band on agarose gel electrophoresis: -, pixel value< 50; +, 50 ~ <110; ++, 110 ~ < 170; +++, 170 ~ < 230. Differential cultivars were Ponkan mandarin (PM), Liucheng sweet orange (LSO), Wentan pummelo (WP) and Eureka lemon (EL).

病菌之傳播與中間寄主

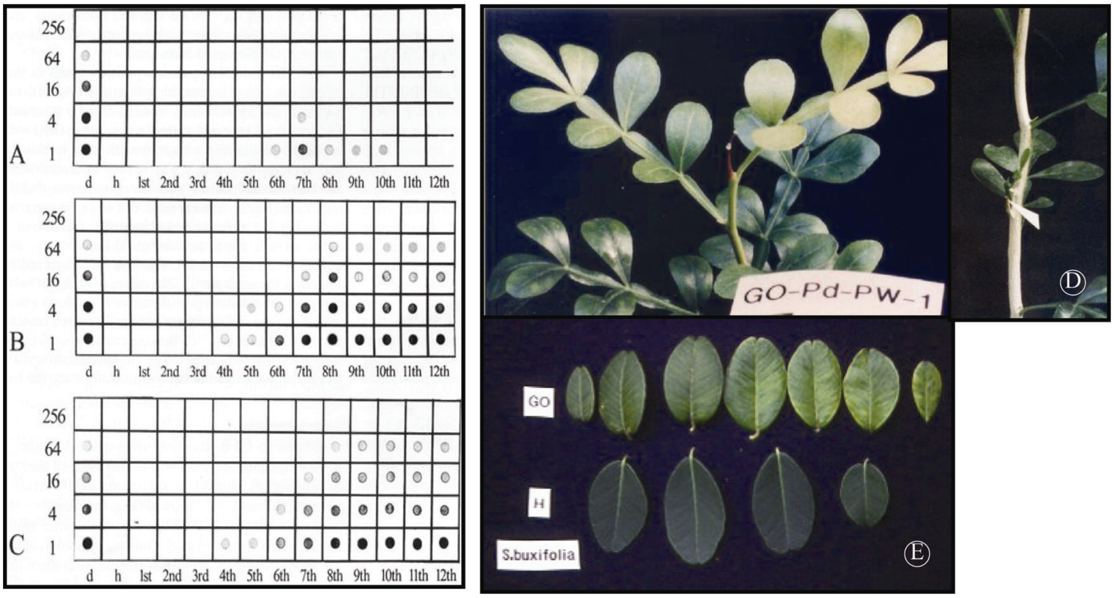
1. 病苗之傳播

此系統性黃龍病主要經無性繁殖之柑桔種苗由人為移動而傳播，而在田間由柑桔木蝨(*Diaphorina citrus*)做永續性之傳染蔓延。台灣的木蝨生態型(Biotype)對黃龍病菌傳播力較低，其成蟲木蝨在罹病柑株上獲毒，傳播率為低於5%而帶毒蟲之傳染力亦低於12.9%。成蟲在病株之獲毒吸汁時間往往超過一天，且若蟲之獲毒率較高。此媒介昆蟲不經卵傳播[1, 19]。用帶病之接穗嫁接繁殖之柑苗，或由染病之母株經高壓繁殖之苗為主要傳播途徑，做長短距離傳播。

2. 中間寄主

柑桔黃龍病除了危害柑桔之外，其他可能性的中間寄主亦成為田間發病生態的注意焦點。因此有四種柑桔木蝨繁殖良好的植物被引起注意，包括烏柑仔(*Severinia buxifolia*)、木蘋果（*Limonia acidissima*）、月橘(*Murraya paniculata* var. *paniculata*)及咖哩葉(*Murraya koenigii*)。將罹病柑株之病芽以人工接種至四種植物上，再以核酸探針點漬雜配法追蹤偵測病菌的存在，發現烏柑仔及木蘋果經人工接種後可偵測到病菌的存在，但月桔及咖哩葉在病原接種後的一年皆無法被偵測到。木蘋果以人工接種病原後在六至十個月期間可偵測到病菌，之後(十一及十二月)即無法偵測到病菌，而其中僅在接種後七個月時病菌濃度為較高(圖五, A)，和病原接種於晚崙西亞甜橙的植株相比，木蘋果中的病原菌僅以低量存在(圖五, A&C)。病原接種六個月後可在植株上觀察到黃化的病徵(圖五, D)，但在一年後植株即回復健康狀。木蘋果中的病原菌因無法長期持續性存在，進入感染數月後又消失，認定為黃龍病暫時性寄主[13, 14]。而病原菌接種於烏柑仔的試驗和接種於晚崙西亞甜橙植株的感染情形相似(圖五, B&C)，在烏柑仔及晚崙西亞甜橙中病原接種四個月後即可偵測到病原菌，且病原濃度較高、病原菌可持續性存在而感染危害植株，在接種後可見烏柑仔有黃化病徵出現(圖五, E)相似於在柑桔上的病徵，因此得知烏柑仔為黃龍病菌良好的寄主[15]。月橘及咖哩葉雖是木蝨生殖最喜好的

寄主，但以核酸探針點漬雜配法及黃龍病專一性引子對(226-primer pair)進行PCR皆無正反應訊號出現[2,4]。顯示台灣之黃龍病菌不會在月桔及咖哩葉繁殖，並非柑桔黃龍病原菌之中間寄主。

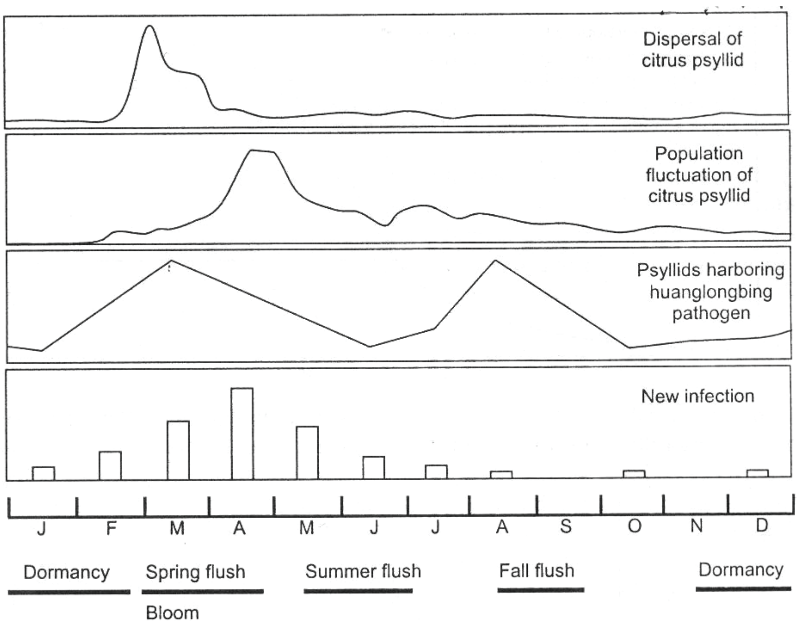


圖五、黃龍病菌在不同中間寄主及甜橙中因抗感性不同之菌體繁殖情況。以點漬雜配法測定病菌繁殖濃度在接種後一年之變動。

Fig. 5. Dot hybridization tests for the detection of HLB-FB in suspected alternative hosts grafted with the HLB-diseased citrus scions, were made monthly within 1 year after inoculation. The total nucleic acid extracted from the test plants of wood apple (A), Chinese box orange (B), and Valencia sweet orange (C) were diluted in four-fold dilution series, and then subjected to dot hybridization. (D) The symptom expression of wood apple (*Limonia acidissima*) graft-inoculated with diseased Ponkan scion. (E) Leaf symptoms of HLB-affected Chinese box orange (*Severinia buxifolia*), alternative host of HLB, showing yellow mottling and hardening of diseased leaves (GO, upper row), compared to healthy green leaves (H, under row).

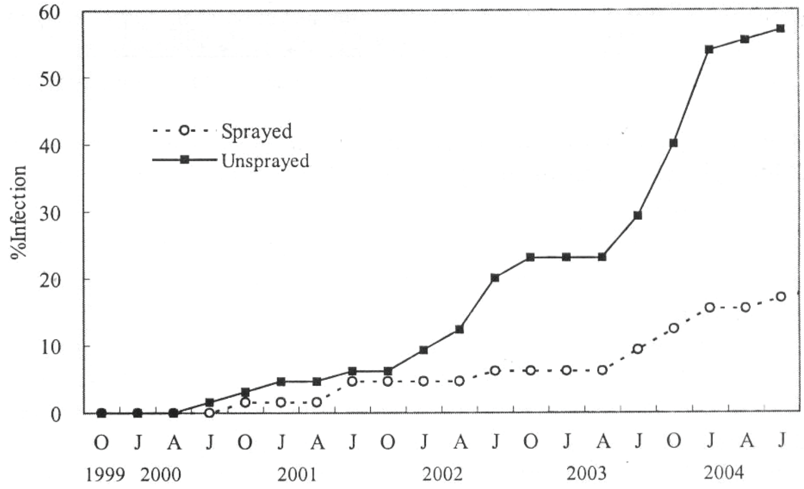
3. 媒介昆蟲之傳播生態

為防阻此病之蔓延，從1983年無病柑桔種苗之生產與栽培就開始推展。健康苗種植於田間後必需防驅木蝨來防阻黃龍病之田間傳播[22, 23]。當傳染源存在而媒介昆蟲密度高時此病傳染流行就發生。病害在田間之蔓延在萌芽期最高，因幼芽供木蝨之取食、繁殖、獲毒與傳播[1]。此病由木蝨之傳播與媒介昆蟲之高密度、帶毒率及遷移飛來量等有正相關[1]。HLB病株群附近之果園中柑桔健樹被傳染發病率增加成S形曲線。以二個月發病潛伏期推最高感染率於嘉義地區為二月下旬至三月中旬，此期間木蝨帶毒率及飛來木蝨之量為高峰期(圖六)。此期間為施藥防驅木蝨之關鍵期。在不施藥防蟲及不掘除病株之果園中柑株感染發病率二年累積達70％。在1999至2004之五年間在南台灣進行黃龍病之傳染率增展試驗，在施藥防蟲試驗區感染率17％，而不施藥區感病達57％(圖七)。



圖六、柑桔木蝨在南台灣果園中傳播柑桔黃龍病之周年情況。

Fig. 6. Phenological patterns, population dynamic and HLBB infection of citrus psyllid in orchards of Southern Taiwan: Dispersal of citrus psyllid, population fluctuation of psyllid, psyllids harboring HLBB and new infection. (Hung, S. C., 2006)



圖七、五年間在噴藥及不噴藥柑園中之黃龍病感染率累積情形。
Fig. 7. HLB progresses of infection rate (%) in sprayed and unsprayed plots during 5 years. The infection cumulative rate of FB infection reached 17% in sprayed plot and 57% in unsprayed put a 5 years later. (Hung, S. C., 2006)

病害診斷與病菌偵測

1. 病害診斷

在果園之病株通常按上述之典型病徵特徵診斷黃龍病。根部病害、天牛為害及肥份營養缺乏等亦引起葉片萎黃病徵，因此需先察看是否此類病蟲害加害。進一步診斷使用罹病性之寬皮柑(如椪柑)或桔橙(如桶柑)等健苗為指示植物，採可疑株之枝條做接穗/芽嫁接接種指示植物，培養於溫室中觀察其發病病徵。病菌在柑株中分佈很不均而密度又低，因此對一樣品需用 2 株以上指示植物，而且一株上要嫁接穗/芽 2 個以上，此生物檢定法頗費時[19]。電顯觀察病組織切片篩管中之病菌體，可確認此病感染[3]。病葉葉肉細胞中澱粉堆積現象，因此可用碘液試劑檢驗病葉係田間快速檢疫法，但敏感度比 PCR 偵測法低。

2. 病菌檢疫偵測

黃龍病於田間主要是以病株葉片與果實的病徵作為判別，進一步的診斷則需將病接穗嫁接於感病的柑株進行生物檢定。由於病原菌於植物

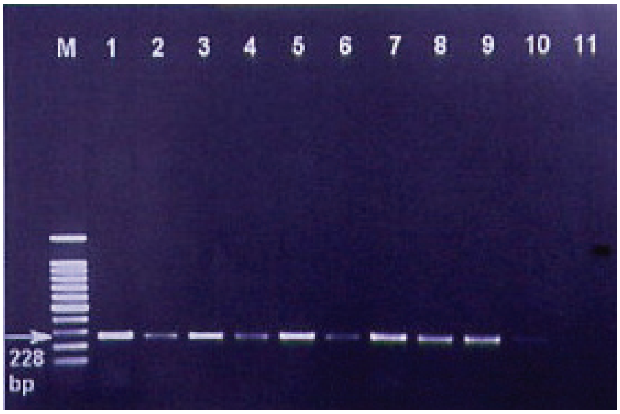
體內常濃度低且分布不均，因此生物檢定需使用較多的試驗植物。嫁接接種經一段時間後再以超薄切片配合電子顯微鏡觀察確認接種植物的韌皮部篩管細胞內是否有病原菌存在，相當耗時費工。因此為能快速診斷，進而開發分子診斷試劑，經病菌基因DNA選殖，獲得具病菌特異性DNA片段，而可成為一非放射性標誌之核酸探針(DNA probe)，再經點漬雜配法測定黃龍病感染與否[2, 14]。而後為更靈敏且快速診斷黃龍病，更進一步將此探針經定序後，從中設計專一性引子(226-primer pair)[2, 13]，建立PCR測定法，配合電泳分析結果。而此專一性引子對國外亞洲型的黃龍病菌分離株亦具反應，包括日本沖繩、中國、馬來西亞、越南、泰國之分離株。而此PCR技術已發展至可偵測黃龍病之寄主植物與媒介昆蟲木蝨[12]，成功應用於病菌偵測與生態的研究，成為快速準確檢疫技術(圖八)。此外PCR測定法也應用於健康種苗生產的檢測。除此之外，由於從電子顯微鏡觀察研究發現受黃龍病菌感染的病組織之葉肉細胞及韌皮部之薄壁細胞皆有澱粉粒之堆積，因此此一特性亦可利用於此病害診斷，而再創製碘液簡易檢疫法檢測黃龍病，並完成簡易的HLB碘試劑檢測套組(Iodine kit)，並標準化(圖九)：以粗細度160~200C之砂紙片削取成熟葉上表面組織，放入PE封口袋，加1cc純水，揉成suspension，再加入1~2滴碘液 (KI 3%+I_2 1.5%)，即呈紫黑色反應，1分鐘內完成反應，目讀檢驗結果，此法廉價簡便，農民易使用，而可於田間進行初步診斷。

黃龍病之健康管理

1. 無病柑桔種苗之體系建立與生產

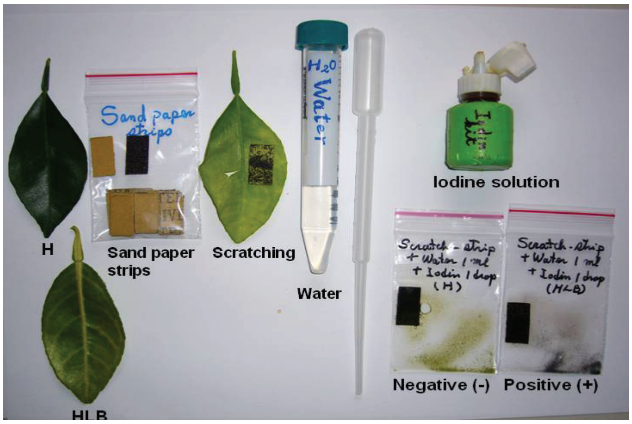
柑桔黃龍病之發生流行於熱帶、亞熱帶地區，現今對柑桔生產構成嚴重威脅。因系統性黃龍病及病毒病害主要由營養繁殖體傳播而在田間經媒介昆蟲迅速傳染蔓延，綜合病害管理(IPM)被推薦於防治此類病害。IPM 包括無病柑苗之繁殖，傳染源之撲滅以及經媒介昆蟲之驅除來預防第二次傳染。無病柑桔種苗體系之建立(PFNS)對此病發生之預防是重要基本措施，在台灣從 1983 起開始著手推展。改進頂梢微體嫁接法(STG)

併用熱療法用於生產無病柑桔原種。精確而快速檢疫技術之研發對無病種苗體系之管理是不可或缺。在台灣 PFNS 包括下列階段：1)STG，2)無病柑桔原種之建立，3)無病柑苗繁殖圃，4)種苗健康驗証制度經檢疫措施執行。PFNS 及健康種苗驗証措施推行目前經台灣大學植微系、嘉義農業試驗分所以及農委會防檢局分工合作推展。



圖八、黃龍病菌之 PCR 偵測，以電泳膠體分析之結果。

Fig. 8. Detection of HLB pathogen by polymerase chain reaction (PCR) followed with electrophoresis analysis: 1~4, Taiwan HLBB isolates; 5, Okinawa isolate, Japan; 6, Chain isolate; 7, Malaysia isolate; 8, Vietnam isolate; 9, Thailand isolate; 10, Saudi Arabia isolate; 11, Healthy CK.



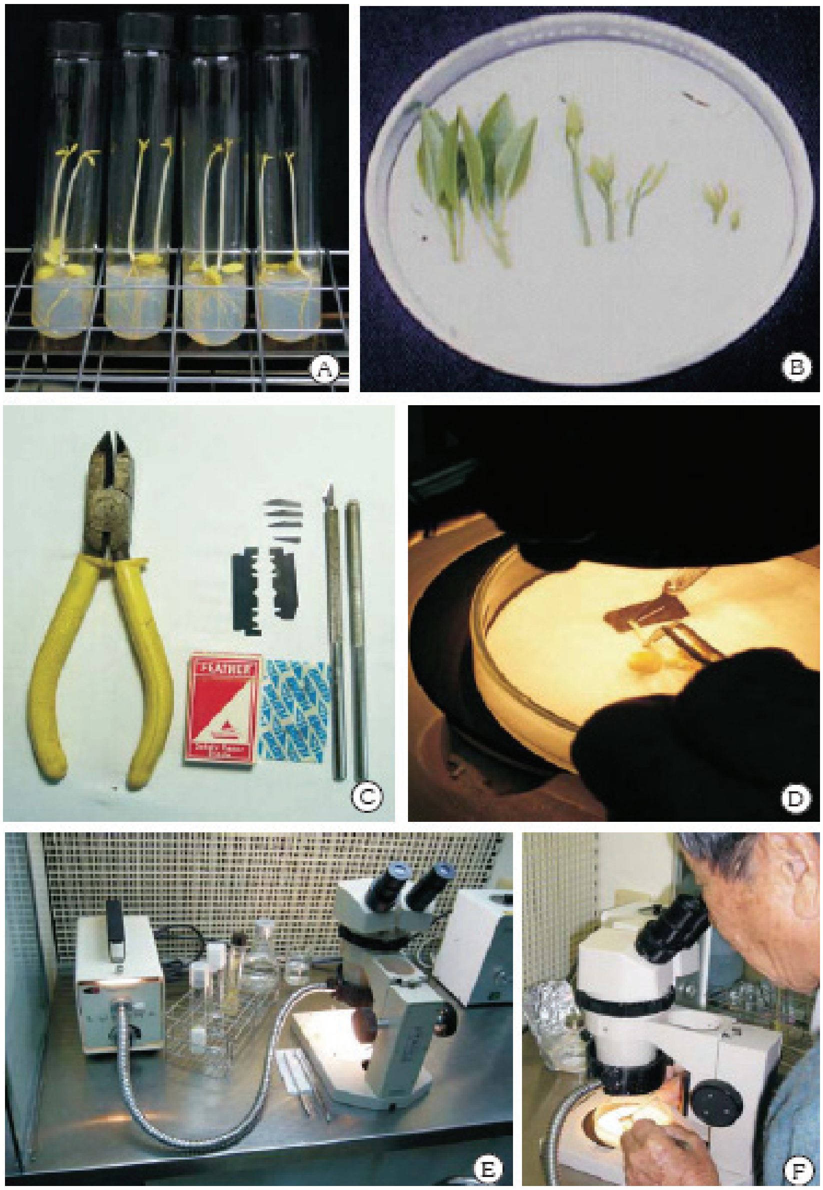
圖九、黃龍病菌之碘液試劑檢疫方法。

Fig. 9. Indexing of HLB infection with Iodine kit including Iodine solution, piece of sand paper, PE bag and pure water: Positive reaction (+) showing color turning to dark brown to black; Negative reaction (-) showing yellow.

1) 頂梢微體嫁接(STG)與無病柑桔原種圃

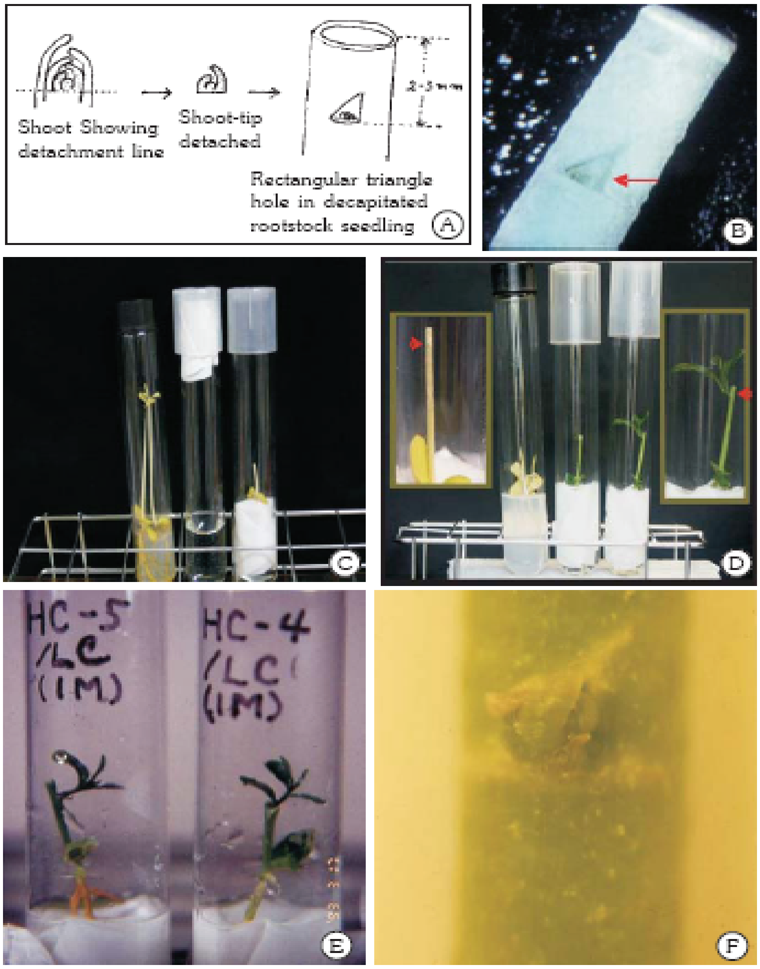
STG 法是從帶病柑桔母樹枝條獲得無病繁殖體之最可靠方法。幼芽頂梢或腋芽生長點組織通常不帶 HLB 病菌與病毒類，而由頂梢生長點組織再生植株通常不含此系統性病原。由一般 STG 法[21]將其倒 T 形嫁接切口修改為直角三角形小穴而為改進 STG 法，可提高 STG 成功率[25]。STG 之操作過程在無菌箱內進行，所用工具及材料(圖十)以及改進 STG 法過程(圖十一)詳細解說記載於作者最近著作小冊[23]。雙層嫁接法開發於促進 STG 株之嫁接生長點組織快速生長，其操作技術過程簡述於下(圖十二)。(1)STG 嫁接苗在 28℃生長箱培育一個半月左右嫁接生長點組織在白化砧苗之三角穴中癒合完成或開始生長後取出試管外，其嫁接上端 1 公分左右切斷供做接穗。(2)將此幼接穗旁接於預先培育於鉢中生長好之砧木苗，嫁接用蠟薄條包綁幼 STG 接穗下部，露出嫁接穴，上端用 PE 小封口袋覆蓋預防嫁接穗乾枯。(3)雙層嫁接株可置放於 28℃生長箱或溫室中培育 3-4 星期後移開 PE 袋，約 3-4 個月後 STG 生長點組織生長出新芽發育成新枝條，經 PCR 檢疫確定無病者，可供做接穗枝條做進一步嫁接繁殖。(4)無病 STG 苗與雙層嫁接苗其生長枝條每隔 3-4 月可供為快速繁殖之嫁接用接穗。

STG 苗經檢驗確定不含 HLBB 及 CTV, CTLV 及 CEVd 等病菌者可供為無病原種株，保存於無病原種圃，設立於嘉義農業試驗分所。原種圃設在不銹鋼網建構之防蟲網室中。該網室具有防蟲侵入雙層門，外圍由水溝環圍(圖十三, A, B&C)。在第一門上裝有電風簾以阻擋昆蟲隨人侵入。室設水泥台於覆蓋粗碎石地板上，走道為水泥路(圖十三, D&E)。原種株通常嫁接於枳橙砧上(Troyer, Carrizo)，每品種保存有 2~4 株。原種株培育於大鉢介質土中，適當剪定促使小量結果，觀察品種園藝特性之變異與遺傳穩定性。淘汰 off-type 變異株。原種株之枝條供為接穗株之繁殖培育。原種株受定期檢疫而檢查果實之正常性。柑桔原種保存於公立試驗或學術機構、防蟲網室或溫室中，國家無病柑桔原種圃設在嘉義農業試驗分所溪口站水田區中。



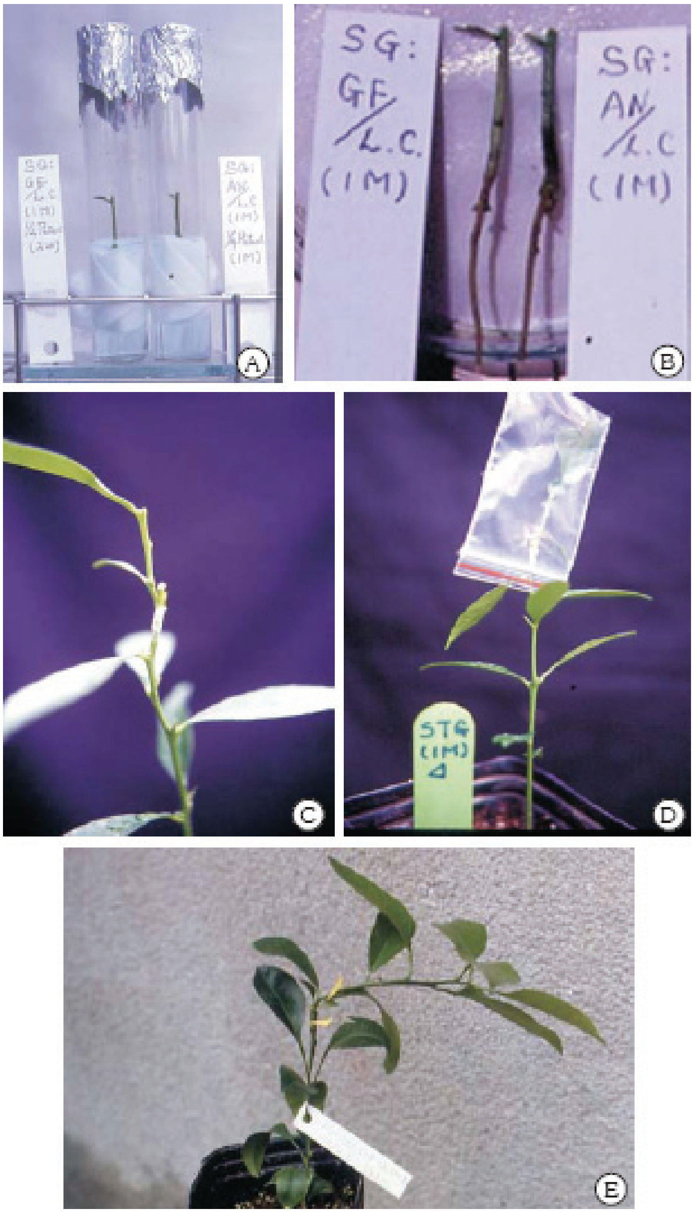
圖十、頂梢微體嫁接之過程：材料與工具。

Fig. 10. Procedure of shoot-tip micrografting. (A) Culture of rootstock in solid medium. (B) Citrus shoots of different sizes for STG. (C) Cutting knife consists of cutting edge of razor blade and edge holder; pliers for making blade edge. (D) Making STG incision on upper top of rootstock seedling with tip-bended forceps and cutting knife. (E) Cold beam illuminator, binocular microscope and accessories within a laminar-flow bench. (F) Making STG incision on upper top of rootstock seedling with tip-bended forceps and cutting knife.



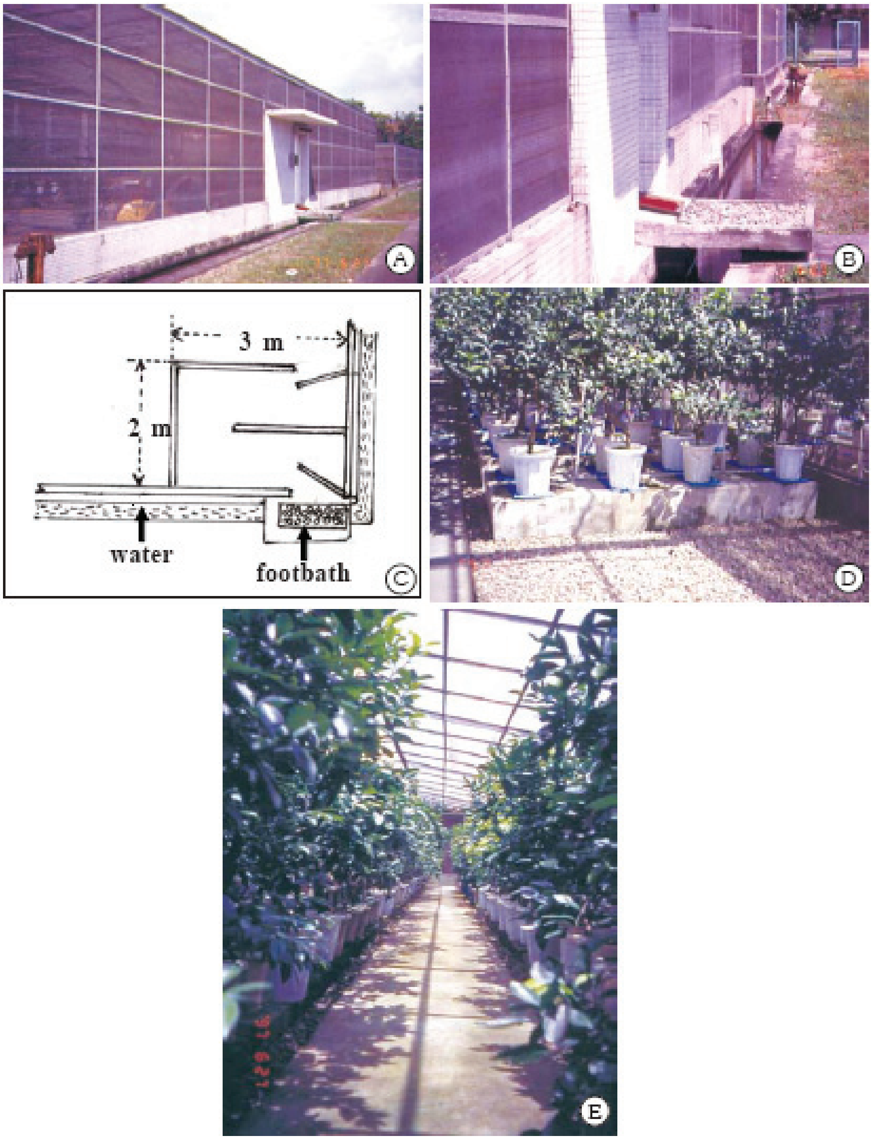
圖十一、頂梢微體嫁接之過程：白化苗與嫁接苗培養，直角三角穴之STG過程圖解。

Fig. 11. Procedure for shoot-tip micrografting. (A) Diagram showing excision of shoot-tip with 2-leaf primordia and rectangular triangle hole (0.3~0.5 mm) on a decapitated rootstock seedling by removing cortex layer with cutting edge of STG knife. (B) A shoot tip placed in the hole on a decapitated rootstock seedling. (C) Two-week old rootstock seedlings in solid medium (left), a sterile test tube with a center-perforated filter-paper platform containing liquid medium (center), and a test tube containing the micrografted rootstock seedling supported by filter paper platform on liquid medium (right). (D) Different stages of STG rootstock seedlings, also showing a new sprout regenerated from the grafted shoot-tip (right). (E) Two new shoots of Hong China sweet orange regenerated from ST in rectangular hole (left) and in V-shaped incision (right) of rootstock seedlings one month after micrografting. (F) A new sprout from ST in rectangular hole on rootstock.



圖十二、STG苗之雙層嫁接法以加速嫁接芽之萌芽與生長。

Fig. 12. Procedure of double grafting with micrografted rootstock seedlings as scion. (A) Sprouting of micrografted seedlings in test-tube culture. (B) Two STG seedlings with sprouts taken out from test tubes for secondary grafting. (C) A potted vigorous rootstock seedling side-grafted with a scion from the STG-seedling. (D) The grafted part of rootstock seedling covered with a mouth-sealed plastic bug. (E) A new mature twig grown from the double grafted rootstock three months after double grafting.



圖十三、國家無病柑桔原種圃網室之結構。

Fig. 13. National repository for maintaining certified pathogen-free citrus foundation blocks. (A) Insect-proof screen-house surrounded by water canal and with double doors. (B) Close-up picture of the entrance with double doors. (C) Drawing of an entrance with double door and footbath containing gravel mixed with copper sulfate solution. (D) Inside view of the screen-house showing healthy foundation trees on concrete benches on gravel floor. (E) Certified budwood increase trees grown in a screen-house attached to the foundation depository.

2) 無病柑桔苗之生產與驗証

柑桔黃龍病在全台低海拔及平地柑桔果園一直普遍發生危害，唯以無病柑苗更新病園才可更生台灣柑桔產業。更種無病苗而推行適宜健康與栽培管理，可望促進永續柑桔產業之發展。因此生產與栽培無病而高品質之柑苗對 HLB 管理係首要基本對策。至今國內生產無病柑苗產量減少，不達所需要量之一成。無病柑苗生產事業之推展與檢驗管理期待促進。

無病苗生產之基本無病種圃完善建立已久，目前由嘉義分所之接穗培質株圃提供無病接穗尚不夠量。由民間合格無病苗圃設立無病接穗繁殖苗室，從無病原種提供種原，其採穗使用期限限定五年，之後得用原種更新。農委會防檢局於 2004 年 12 月 31 日公佈「柑桔無特定病蟲害驗証作業須知」，法令規範無病柑桔種苗之無病原種、繁殖苗及無病苗之生產及檢疫檢驗辦法，並規定檢疫流程與方法，由此法規定設立民間無病苗圃(圖十四)按辦法生產無病苗，受定期檢疫輔導，無病苗出貨前需經抽檢、檢疫合格後發行驗証合格証貼標始得出售。

無病苗以介質植鉢生產。健康砧木苗在網室內生產。砧木苗栽育在含消毒過介質土之苗管(徑 5 ×高 18 cm)或育苗槽中，砧木苗達 30 公分左右移植到內含消毒介質土之打孔塑膠袋內(徑 10×高 30 cm)，苗高超 40 公分可供嫁接，嫁接方法可參考快速繁殖嫁接法[23]。使用美工刀嫁接無病芽或穗，以芽接或旁接法(side-graft)，於砧木苗距土面 25-30 公分處嫁接，用蠟膜帶裹綁芽/穗，在接點上接芽方向反折，待芽穗萌芽超出 5 公分後由接點上方剪除砧木莖，並時常巡視摘除腋芽以促進接穗萌長。在肥培管理良好下芽接 3-4 個月後苗高可達 50 公分以上，可出貨田間定植。在育苗中注意疫病、根瘤線蟲及潰瘍病之發生，而嚴格防阻媒介昆蟲之侵入。砧木品種之選擇要注意穗砧之親和力與抗病性。一般酸桔及美人柑用於寬皮柑，Troyer 及 Carrizo 接於橙類及寬皮柑；苦柚及 Swingle 枳柚用於柚類。廣東檸檬及 Volkamer 砧常用於各種柑桔類。

圖十四、柑桔無病苗圃網室，無病砧木苗及嫁接苗之缽植栽培。

Fig. 14. Production of PF citrus seedlings in screen houses by private nurseries. (A) Cultivation of rootstock seedlings in seedling tubes (arrow), the secondary transplanting in large plastic containers (upper left plants), and budded plants prepared by grafting clean bud or scion onto rootstock seedlings (right). (B) A well-grown budded nursery plant in container, ready for transplanting to the field.

圖十五、無病柑桔苗種植於田間果園後良好之發育與結果。

Fig. 15. Rehabilitation of citrus orchards planted with PF seedlings.

Cultivation of pathogen-free pummelo seedling (A) and Ponkan mandarin seedlings (B) in orchard. Two-year-old PF Wentan pummelo and Ponkan tree began fruiting. Dr. Garnsey and author stand by Ponkan tree in the center (B). (C) Luxuriant growth of 3-year-old sweet orange trees in an orchard planted with PF seedlings. (D) Normal bearing of fruits of high yield on the 3-year-old PF trees in the orchard as shown in the picture (D) with the author and grower.

2. 無病柑桔苗之種植與健康管理

為防治黃龍病與病毒病害，推行無病柑苗來更新生產。無病苗種植田間後比一般柑苗生長快而種植後 2 年開始結果達相當量且品質良好，若做適宜之健康管理及肥培管理，例如適宜之灌溉水，施肥與剪定(圖十五)。至今無病苗之推廣不夠普及，今後仍待公家與民間合力推廣獎勵。至於無病苗種植果園後需勵行下列策略與健康管理：

1) 傳染源之撲滅：隨時掘除 HLB 病株及中間寄主植物以避免此傳染源經媒介昆蟲傳播到鄰近之健康柑樹。

2) 媒介昆蟲驅除：施用殺蟲劑於柑樹保護防阻媒介昆蟲傳病感染。可在萌芽期即傳病危險期(2-3 月)噴施適當殺蟲劑(例如大滅松, Confidor)以防驅木蝨與蚜蟲。已引進之木蝨天敵寄生峰(*Tamarixia radiate*)定著於本島，生物防治木蝨已達效果。

3) 果園隔離：果園周圍種植防風林對隔離媒介昆蟲飛進園內有所助益。將果園設立於遠離疫區之新地區亦有隔絕效果。

4) 病株之抗生素灌注藥療：於田間病株的化學療法上，部份柑橘生產者曾以 1976 年(Su 與 Chang)所報告的方法灌注四環黴素以治療病株。近來灌注效率則可以加壓灌注器(圖十六)取代早期重力點滴法獲得改進，抗生素灌注之方法程序與注意事項列於表二。於灌注器內加入高壓 (壓縮空氣)在 80 磅下，灌注 1000 ppm 之四環黴素(2~4 公升/每棵) 3 次(秋 2 次，春 1 次)，可達良好藥療效果，處理後病株長出健康新生葉，產生好品質柑果[4]。注射後，剪定頂梢枯枝可增進治療效果。此法常引起暫時性藥害包括葉片焦枯、葉脈褐化、大量落葉與新生葉細長，但可於半年之後恢復正常生長[4]。此治療法建議病株於發病早期(病葉比率占樹冠一半以下)處理。

於2004年3月HLB初發現於巴西聖保羅兩農場，到2006年10月，2年半間此病蔓延到3000個農場中65萬株柑樹，可見傳播速度之迅速。當時也發現月桔被HLB感染也能做傳染源。從2005年7月起該國法令執行掘除柑桔病株[16]。至2009年11月，5年半內有200萬株柑樹檢查到感染此病，因此掘除周邊之罹病柑樹共砍除400萬株。該國體驗此事實認定HLB管理有下列二原

則：(1)傳染源之撲滅，HLB病株之隨時掘除,(2)殺蟲劑之噴灑防驅媒介昆蟲木蝨，HLB能以此二原則來防治[5]。此對策原則可供吾方參考。

圖十六、抗生素之灌注藥療：工具與壓力灌注。
Fig. 16. Chemotherapy of HLB-diseased citrus tree by antibiotic injection. (A) Injection of 1~4 L of 1000 ppm Achromycin with a air-pressured injector to the basal portion of trunk. (B) A cordless battery-driving drill (Bosch), and a wood-type needle for drilling injecting hole. (C) Diagram of air-pressured plastic injector.

表二、抗生素灌注之方法程序與注意事項

Table 2.Antibiotic injection schedules and cautious

A. Formula:

(1) 1000 ppm of Achromycin (Tetracycline) :
1 g Achromycin in 1 litter clean water

(2) 1000k IU of Penicillin-G :
2 bottles of Pencillin-G (1,500,000 IU) in 3 litters of clean water.

B. Dosage of antibiotic solution:

Diameter of trunk (cm)	<10	11~20	21~30	>31
Volume of solution (L)	1	2	3	4

C. Application cycles:

(1) 2 applications of Achromycin solution at 2~3 weeks interval after fruit harvest (mainly in early spring in Taiwan)

(2) 1 application of Achromycin followed by 1 application of Penicillin-G at 2 week interval.

(3) 2 applications of Achromycin followed by 1 application of Penicillin-G at 2 week interval.

D. Site of injection:

Injection holes of 3 cm deep, are made at 10~20 cm from soil level on the swelling site of trunk on diseased-branch side.

E. Cautions of injection:

(1) Injection is made in the forenoon of sun-shining day.

(2) Irrigation of water has to be done just after injection in dry soil.

(3) Pruning off the diseased twigs or branches 1 week after the last injection.

(4) Injection pressure within an injector at 80 lbs.

(5) Treatment of injection hole with pruning protectant paint e.g. wound paste (Topsin M) or sick solution of copper oxychloride + metalaxyl (100X).

(6) A injection recommended for curing HLB-diseased trees at early stage of disease (<50％ diseased twig).

(7) Injection time : just before sprouting time or after harvest (spring , autumn).

結論與展望

柑桔黃龍病起因於不能培養之特殊細菌，係系統性似病毒病害，主要經種苗傳播，在田間由木蝨傳播蔓延，易流行發生成災。此病通常以綜合防治法防治。無病種苗之生產體制建立係首要對策。病菌偵測技術已建立，對無病種苗之檢疫及健康管理有助益。吾國無病種苗生產與驗証制度早已建立完成，唯未普及推行，無病苗對HLB管理及產業更新尚未成效。無病苗定植後田間健康管理仍待改進與推行。其中病株傳染源之撲滅需要徹底執行，而媒介木蝨之藥劑驅除對此病之防治亦是基本原則。今後此病之進一步流行病學研究可供為HLB管理改進之依據。至今尚未發現具有此病的抗性品種，仍待今後遺傳工程或一般育種育成抗病性品種，以抵擋柑桔黃龍病之侵襲。

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引用文獻

1. 洪士程 2006 柑橘木蝨傳播黃龍病之生態研究。台灣大學昆蟲學研究所 博士論文。164 頁。
2. 洪挺軒 1994 柑橘黃龍病原擬細菌診斷用核酸探針之製備與應用於感

染生態之研究。台灣大學植物病蟲害研究所 博士論文。258 頁。

3. 黃安利 1987 柑橘立枯病原菌之形態與消長動態之電顯研究。台灣大學植物病蟲害研究所 博士論文。148 頁。
4. 蔡佳欣 2007 柑橘黃龍病之病菌系統、發病生態與化學治療。台灣大學植物病理與微生物學研究所 博士論文。150 頁。
5. Belasque Jr, J. *et al*. 2010. Lessons from Huanglongbing management in Sao Paulo state, Brazil. Journal of Plant Pathology 92:285-302.
6. Bové, J. M. 2006. Huanglongbing: A destructive, newly-emerging century-old disease of citrus. J. Plant Pathol. 88:7-37.
7. Calavan, E. C. 1968. A review of stubborn ad greening diseases of citrus PP. 105-117. In J. F. L. Childs (ed), Proc. 4th Conf. IOCV University of Florida Press, Gaines ville.
8. Cheng, C. P. 1943. Studies on 'Huanglongbing' of Chaukan tangor. Jour. New Agr. 3:142-172 (In Chinese).
9. Chien, C. C., Chiu, S. C. and Ku, S. C. 1989. Biological control of *Diaphorina citri* in Taiwan. Fruit 44: 401-407.
10. Etienne, J. and Aubert, B. 1980. Biological control of psyllid vectors of greening disease or Reunion island. PP. 118-121. In Calavan, E. C., S. M. Garnsey and L. W. Timmer. (eds.) Proc. 8th Conference of IOCV, University of California, Riverside.
11. Hamashima, A. *et al.*, 2003. First report of citrus greening disease in Kagoshima. Ann. Phytopathol. Soc. Jpn. 69: 307-308.
12. Hung, T. H., Hung, S. C., Chen, C. N., Hsu, M. H. and Su, H. J. 2004. Detection by PCR of *Candidatus* Liberibacter asiaticus, the bacterium causing citrus huanglongbing in vector psyllids: application to the study of vector-pathogen relationships. Plant Pathology 53: 96-102.
13. Hung, T. H., Wu, M. L. and Su, H. J. 1999. Development of a rapid method for the diagnosis of citrus greening disease using the polymerase chain reaction. J. Phytopathology 147, 599-604.

14. Hung, T. H., Wu, M. L. and Su, H. J. 2000. Identification of alternative hosts of the fastidious bacterium causing citrus greening disease. J. Phytopathology 148, 321-326.
15. Hung, T. H., Wu, M. L. and Su, H. J. 2001. Identification of the Chinese box orange (*Severinia buxifolia*) as an alternative host of the bacterium causing citrus Huanglongbing. European Journal of Plant Pathology 107: 183-189.
16. Lopes, S. A. 2006. Huanglongbing in Brazil. PP.11-19. Intern. Workshop: Prevention of Citrus Greening Disease in Severely Infected Areas. TARRI JIRCAS, Ishigaki, Japan.
17. Lopes, S. A., Bassanezi, R. B., J. Belasque, Jr., Yamamoto, P. T. 2008. Management of citrus Huanglongbing in the State of Sao Paulo-Brazil. PP. 107-117. Proc. FFTC-PPRI-NIFTS Joint Workshop: Management of Citrus Greening and Virus Disease for Rehabilitation of Citrus Industry in the ASPAC. Hanoi, Vietnam.
18. Matsumoto, T., Su, H. J. and Lo, T. T. 1968. Likubin. PP 63-67. In USA Agriculture Handbook No. 333. Indexing Procedures for 15 Virus Disease of Citrus Trees.
19. Matsumoto, T., Wang, M. C. and Su, H. J. 1961. Studies on Likubin. In W. C. Price (ed.) Proc. 2nd Conference of the International Organization of Citrus Virologists (IOCV). University of Florida Press. Gainesville. PP. 121-125.
20. Miyakawa, T. and Tsuno. 1989. Occurrence of citrus greening disease in the Southern islands of Japan Annals Phytopathological Society of Japan. 55:667-670.
21. Murashige, T., Bitters, W. P., Rangan, T. S., Roistacher, C. N. and Holliday, B. P. 1972. A technique of shoot apex grafting and its utilization towards recovering virus-free citrus clones. HortScience 7:118-119.
22. Su, H. J. 2006. The current measures and perspective of controlling citrus

greening disease (HLB) in Taiwan (Abstract) P30 Proc. The International Workshop for Prevention of Citrus Greening Disease in Severely Infested Areas. Dec. 2006, TARF/JRCAS, Ishigaki, Japan.

23. Su, H. J. 2008. Production and Cultivation of free Citrus Saplings for Citrus Rehabilitation in Taiwan. pp 51. Published by Asia-Pacific Consortium on Agricultural Biotechnology and Asia Pacific Association of Agricultural Research Institutions (APAARI) C/O FAO Regional Office for Asia and the Pacific, Bangkok, Thailand.
24. Su, H. J. 2008. Research and health management of citrus Huanglongbing in Taiwan. PP. 57-92. Proc. International Research Conference on Huanglongbing. Orlando, Florida, USA. (http://www.doacs.state.fl.us/pi/hlb_conference/events.html)
25. Su, H. J. and Chu, J. Y. 1984. Modified technique of citrus shoot-tip grafting and rapid propagation method to obtain citrus budwoods free of citrus viruses and Likubin organism. Pr.c. Int. Soc. Citriculture 2:332-334.
26. Su, H. J. and Chang, S. C. 1976. The response of the Likubin pathogen to antibiotics and heat therapy. PP. 27-34. In E. C. Calavan, (ed). Proc. 7th Conference of IOCV, University of California, Riverside.
27. Su, H. J. and Matsumoto, T. 1972. Further study on the pathogen complex causing Likubin of citrus in Taiwan. PP. 28-34. In W. C. Price (ed). Proc, 5th Conference of IOCV University of Florida Press, Gainesville.
28. Tomimura, K. *et al*., 2009. Evaluation of genetic diversity among "*Candidatus* Liberibacter asiaticus" isolates collected in Southeast Asia. Phytopathology 99: 1062-1069.
29. Tsai, C. H., Hung, T. H. and Su, H. J. 2008. Strain identification and distribution of citrus Huanglongbing bacteria in Taiwan. Botanical Studies 49: 49-56.

ABSTRACT

The Development and Effective Use of Pathogen-free Seedling as the Main Component of IPM of Citrus Huanglongbing Disease

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Citrus Huanglongbing (HLB) locally called "Likubin" was fist identified in Taiwan in 1951, and became seriously epidemic over the island in 1956. Thenafter, Matsumoto and his colleagues started etiological study on the disease and clarified the transmissible nature as a virus-like disease and finally found phloem-limited fastidious bacteria with pleomorphic morphology to be associated with HLB. Nowadays, the tropical form of psyllid-borne HLB pathogen has been nominated as *Candidatus* Liberibacter asiaticus. So far, the authors identified four strains of HLB pathogen in Taiwan. The greening disease commonly with mix-infection of citrus tristeza and/or tatter leaf viruses, causes sever yellow mottling and decline, and ultimately death of citrus tree. These systemic diseases are generally controlled by the integrated control measures including cultivation of pathogen-free seedlings, elimination of inoculum sources and prevention of secondary spread by controlling vector insects. Establishment of pathogen-free nursery system is primarily important for preventing prevalence of these diseases. The pathogen-free nursery system has been properly performed through improved techniques of shoot-tip micrografting for obtaining virus/HLB-free citrus foundation stocks, and then production of healthy citrus seedlings within insect-proof screen house under health certificate regulation in Taiwan. The precise and rapid indexing techniques are indispensable for management of pathogen-free nursery system

and health management of citrus tree in the field. Production and cultivation of pathogen-free and high-quality nursery trees are fundamentally important component of HLB management. A healthy citrus orchard planted with pathogen-free seedling may outlive the grower. Healthy citrus trees have a great potential for sustainable high yield over many decades, provided appropriate horticultural and disease management practices are followed.

Key words: Citrus Huanglongbing、HLB strain、health management、pathogen-free nursery system